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# GRADIENT ELUTION IN LIQUID CHROMATOGRAPHY

## III. VERIFICATION OF THE THEORETICAL RELATIONSHIPS FOR ELUTION CHARACTERISTICS (RETENTION VOLUME, BAND WIDTH AND RESOLUTION) IN ISOCRATIC AND GRADIENT ELUTION CHROMATOGRAPHY ON SILICA

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SUMMARY

Theoretical assumptions concerning the influence of the composition of binary mobile phases on capacity ratios in adsorption chromatography were verified using model experiments with chromatography of four N,N-dimethyl-*p*-aminobenzeneazobenzoylamides on columns packed with silica (Porasil A) in binary mobile phases composed of ethyl acetate and cyclohexane. In this system, good agreement was found between the experimental values of the elution characteristics (retention volume, peak width, resolution and number of theoretical plates) in isocratic and gradient elution chromatography and the values calculated according to the theoretical relationships derived in Parts I and II of this series. Twenty different gradient functions (linear, convex and concave) were tested and the agreement between experiment and theory was found to be satisfactory in all cases.

## INTRODUCTION

In Parts I and II of this series[1,2], a theory was presented that made possible the estimation of the elution characteristics in isocratic and gradient elution liquid chromatography and in chromatography involving elution with several steps that differ from each other in the shape of the concentration gradient function. This theory is based on two simple basic equations that describe the relationship between the concentration (molar) of the more efficient eluting component in the binary mobile phase ($c$) and the capacity ratio of a sample compound ($k'$):

$$k' \approx k'_0 \cdot c^{-n} \tag{1}$$

and

$$k' \approx k'_0 \cdot 10^{-n \cdot c} \tag{2}$$

where $k'_0$ and $n$ are constants.

Eqn. 1 can be used in adsorption and ion-exchange chromatography and eqn. 2 in partition chromatography, including salting-out chromatography and chromatography on ion exchangers in aqueous organic media. Theoretical limitations of the validity of these two equations were discussed in Part I[1]. Relationships were derived between the retention volume, retention ratio (separation factor), peak width and resolution and the concentration of the more efficient eluting component in the binary mobile phase in isocratic elution chromatography, in which the mobile phase has a constant composition during the elution.

In Part II[2], eqns. 1 and 2 were utilized in the derivation of the equations for retention volume, retention ratio, peak width, resolution and number of theoretical plates in gradient elution chromatography and in chromatography with several different elution steps. In this derivation, it was assumed that the composition of the mobile phase does not change during the transport from the outlet of the gradient-generating device to the top of the column and that the gradient-generating device is able to produce any required mathematical form of the concentration gradient (for example, a gradient pump based on a photoelectric curve-follower). The equation

$$c = \left(A^{\frac{1}{\varkappa}} + B \cdot V\right)^{\varkappa} \tag{3}$$

was chosen to define the concentration gradient (the relationship between the concentration, $c$, of the more efficient eluting component in the binary mobile phase at the outlet of the gradient-generating device and the volume of the eluate, $V$). In eqn. 3, $A$, $B$ and $\varkappa$ are constants that characterize the initial concentration at the beginning of the gradient elution ($c_0$), the steepness (slope) and the shape (curvature) of the gradient function.

The parameters $A$ and $B$ are defined as follows:

$$A = c_0 \tag{3a}$$

and

$$B = \frac{c_k^{\frac{1}{\varkappa}} - c_0^{\frac{1}{\varkappa}}}{V_y} \tag{3b}$$

where $V_y$ is the volume of the eluate where $c = c_k$; an arbitrary value can be chosen for $c_k$ (it is advantageous to put either $c_k = 1$ or $c_k = 0$).

In this paper, the results of the verification of eqn. 1 and of the equations for elution characteristics in both isocratic and gradient elution chromatography are presented for the adsorption chromatography of N,N-dimethyl-*p*-aminobenzeneazobenzoylamides on silica in cyclohexane–ethyl acetate binary mobile phases.

## THEORETICAL

The equations derived in Part I[1] and Surveyed below are used for adsorption and ion-exchange chromatography and are based on eqn. 1.

*Isocratic elution*

Retention volume, $V_R$, $V'_R$:

$$V_R \approx V_m \cdot (1 + k'_0 \cdot c^{-n}) \tag{4}$$

$$V'_R \approx V_m \cdot k'_0 \cdot c^{-n} \tag{4a}$$

Peak width, $w$:

$$w \cong \frac{4\,V_m}{\sqrt{N}} \cdot (1 + k'_0 \cdot c^{-n}) \tag{5}$$

Resolution, $R_s$, of two compounds 1 and 2 (for $n_2 \approx n_1 \approx n$):

$$R_s \cong \frac{\sqrt{N}}{2} \cdot \frac{k'_{02} - k'_{01}}{k'_{02} + k'_{01} + 2 \cdot c^n} \tag{6}$$

In these equations, $V_m$ is the volume of the mobile phase in the column (column void volume), $N$ is the number of plates in the column, $c$ is the mole fraction of the more efficient eluting component in the binary mobile phase, $n$ and $k'_0$ are constants in eqn. 1 ($k'_{01}$ and $k'_{02}$ relate to the compounds 1 and 2, respectively) and $k'_0$ is the capacity ratio of the sample compound in the mobile phase where $c = 1$ (pure more efficient eluting solvent).

*Gradient elution*

The following equations were derived for elution using concentration gradient functions which can be expressed by eqn. 3.

Retention volume, $V_{R(g)}$, $V'_{R(g)}$:

$$V_{R(g)} = V_m + V'_{R(g)} \tag{7}$$

$$V'_{R(g)} \approx \frac{1}{B} \cdot \left[ (\varkappa \cdot n + 1) \cdot B \cdot k'_0 \cdot V_m + A^{\frac{\varkappa \cdot n + 1}{\varkappa}} \right]^{\frac{1}{\varkappa \cdot n + 1}} - \frac{A^{\frac{1}{\varkappa}}}{B} \tag{8}$$

Peak width, $w_{(g)}$:

$$w_{(g)} \approx \frac{4\,V_m}{\sqrt{N}} \cdot \left\{ 1 + k'_0 \cdot \left[ A^{\frac{1}{\varkappa}} + B \cdot (V'_{R(g)} - V_z) \right]^{-\varkappa \cdot n} \right\} \tag{9}$$

Plate number, $N_{(g)}$:

$$N_{(g)} = 16 \cdot \left( \frac{V'_{R(g)} + V_m}{w_{(g)}} \right)^2$$

$$\approx N \cdot \left( \frac{V'_{R(g)}}{V_m} + 1 \right)^2 \cdot \frac{1}{\left\{ 1 + k'_0 \cdot \left[ A^{\frac{1}{\varkappa}} + B \cdot (V'_{R(g)} - V_z) \right]^{-\varkappa \cdot n} \right\}^2} \tag{10}$$

Resolution, $R_{s(g)}$, of two compounds 1 and 2:

$$R_{s(g)} = 2 \cdot \frac{V'_{R(g)2} - V'_{R(g)1}}{w_{(g)2} + w_{(g)1}} \qquad (11)$$

In these equations, $A$, $B$ and $\varkappa$ represent constants in eqn. 3 (adjustable parameters of the concentration gradient), $N$ is the number of plates in the column in isocratic elution chromatography under otherwise identical conditions, $V_z$ is the volume of the connecting tubing between the outlet of the gradient-generating device and the top of the column and $V'_{R(g)1}$, $V'_{R(g)2}$, $w_{(g)1}$ and $w_{(g)2}$ are the retention volumes and peak widths of sample compounds 1 and 2, respectively.

## EXPERIMENTAL

### *Instrumentation*

The liquid chromatograph used is shown schematically in Fig. 1. The instrument consists of a gradient pump (5) based on a photoelectric curve-follower (PPM-

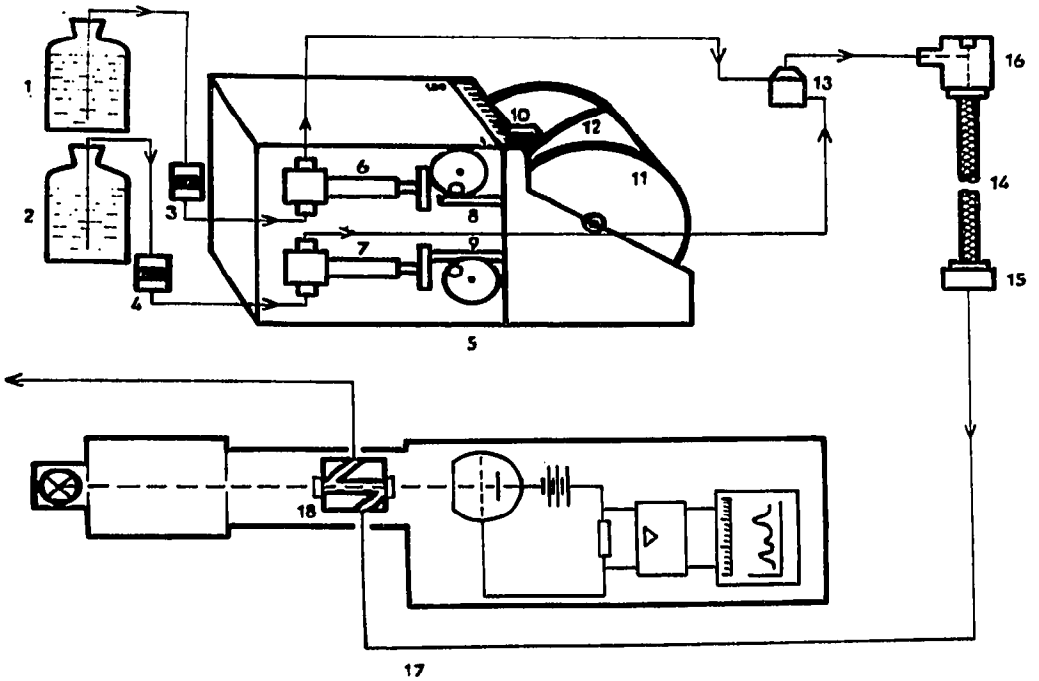


Fig. 1. Schematic diagram of the liquid chromatograph used. 1, 2 = Reservoirs of the two components of the mobile phase (cyclohexane and ethyl acetate); 3, 4 = sintered-glass filters; 5 = gradient pump based on a photoelectric curve-follower; 6, 7 = sapphire plunger pumping blocks with valve systems; 8, 9 = rods for adjusting the pumping ratio of the two plunger blocks; 10 = photo-cell of the curve-follower; 11 = rotating drum of the curve-follower; 12 = black trace of the concentration gradient function; 13 = mixing chamber of small volume; 14 = chromatographic column (glass); 15 = brass column end-fitting; 16 = septum sample injection port; 17 = continuously variable wavelength (360–780 nm) spectrophotometric detector; 18 = flow-through detector micro-cell (8 $\mu$l volume).

68005, Workshops of the Czechoslovak Academy of Sciences, Prague), a glass column of our own design (14) and a spectrophotometric detector (17).

The two components of the binary mobile phase are pumped by two plunger blocks (6, 7) from two reservoirs comprising glass bottles of 500-ml capacity (1, 2) through sintered-glass filters (3, 4), which protect the pump against solid impurities, into a mixing chamber of small volume (13). The pumping ratio of two sapphire plungers is adjusted by two rods (8, 9), the positions of which are controlled by a servo-motor actuated by an electrical signal from a photo-cell (10) sensitive to a deviation of its position from the boundary between the white surface of the paper fastened on a rotating drum (11) and the black trace (12) of the graph representing the mathematical function of the concentration gradient. The total flow-rate of the binary mobile phase is maintained constant during the gradient elution.

The glass column (14), 400 mm long and 3.0 mm I.D., is provided with brass end-fittings (15). The fitting on the upper end of the column is screwed into the T-sample injection port (16), consisting of a PTFE body pressed into a stainless-steel sleeve and a silicone-rubber septum fastened in a metal ring and protected against direct contact with the mobile phase by a PTFE insertion foil[3]. The lower end of the column is connected to the detector (17) by means of a short PTFE capillary (0.2 mm I.D.).

A single-beam Spekol spectrophotometer, operating in the range 360–780 nm (Zeiss, Jena, G.D.R.) is provided with an adaptor of our own construction[4], housing a flow-through PTFE measuring micro-cell (18)[5]. The optical path of the micro-cell is 10 mm, its volume 8 $\mu$l and its I.D. 1 mm. The total volume of the micro-cell and the connecting tubing (PTFE, 0.2 mm I.D.) to the lower end of the column is 30 $\mu$l.

The individual parts of the instrument are connected by means of thick-walled PTFE tubing (0.5 mm I.D.). The end of each tube is broadened to form a cone, which is fitted on a screw-plug on the respective part of the instrument and tightened by means of a brass nut to form a leak-proof connection.

The instrument can be operated up to column pressures of about 40 atm with no leakage of the mobile phase. The construction of the injection port permits direct on-column injection with no sample dilution (zero dead volume). The reproducibility of the injection with a standard 10-$\mu$l Hamilton syringe is satisfactory.

The total extra-column contribution to the retention volume (the total transportation lag of the mobile phase) caused by the injection port, detector and the connecting capillary tubing to the column is 40 $\mu$l. The extra-column contribution to the peak broadening, characterized as the half-width of the peak of the substance injected into the system without the column (the injection port connected directly to the detector) is about 200 $\mu$l at a flow-rate of 2 ml/min and about 130 $\mu$l at a flow-rate of 0.2 ml/min. These contributions to the retention volume and peak width are sufficiently low to be neglected in the present work. (It can be shown that these contributions to a peak of a non-retained compound represent an increase in retention volume ($V_R' = 0$) of about 2% and in peak width ($w = 0.68$ ml) of about 8% under the conditions used, *i.e.*, a column packed with Porasil A, 400 × 3 mm; $V_m = 2.00$ ml; flow-rate 38.5 ml/h. These values are even smaller for retained compounds.)

The photometric detector used had good sensitivity, baseline stability and linearity in the range 0–1 absorbance unit.

The gradient pump produced concentration gradients with a reproducibility of

about ±1 % and maintained the flow-rate of the mobile phase constant to within less than 1 %.

No pressure pulsation damping device was used as the column itself damped the pulsations caused by the pump to such an extent that a smooth recording trace was obtained.

The connecting tubing (PTFE, 0.5 mm I.D.) between the mixing chamber of the gradient pump and the injection port of the column has a small volume ($V_z$ = 0.67 ml).

*Operating conditions*

*Column.* Glass, 400 × 3.0 mm.

*Packing material.* Silica: (a) Porasil A (60), 37–75 μm (spherical) (Waters Ass., Milford, Mass., U.S.A.); (b) Kieselgel (Merck, Darmstadt, G.F.R.), irregular particle shape, screened to obtain the fraction under 50 μm, which was separated from fine powder particles by repeated decantation with water.

The adsorbents were activated for 1 h at 110–115° and the columns were dry-packed. The volume of the mobile phase in the column (the column void volume), $V_m$, was determined in independent runs with columns equilibrated with water by measuring the retention volume of a non-retained, water-soluble compound (the azo-dye Ponceau 6R, molecular weight 706), and was 2.00 ml for Porasil A and 1.65 ml for Kieselgel, respectively.

*Mobile phase.* The binary mobile phase was prepared by mixing cyclohexane with ethyl acetate (both reagent grade) directly by means of the gradient pump from the two reservoirs in a constant volume ratio (isocratic elution) or in a volume ratio that changed with time according to the mathematical function selected (gradient elution). As the flow-rate of the mobile phase was independent of the composition of the mobile phase, eventual volume contractions connected with the mixing of the two solvents were considered to be negligible.

The concentration gradient was started at the sample injection time. The time required for the equilibration of the column with the starting solution after the end of the gradient (or with the mobile phase of a different composition in isocratic elution experiments) was about 15–20 min.

*Compounds chromatographed.* A synthetic mixture of four coloured N,N-dimethyl-*p*-aminobenzeneazobenzoylamides, prepared from dimethylamine, diethylamine, di-*n*-propylamine and di-*n*-butylamine[6], was used as a solution in ethyl acetate (*ca.* 1 mg/ml of each compound). The volume of sample injected was 8 μl.

*Detection.* Photometric, 440 nm.

## RESULTS AND DISCUSSION

*Isocratic elution*

Table I gives the experimental values of the corrected retention volumes, $V_R'$, of the four N,N-dimethyl-*p*-aminobenzeneazobenzoylamides on a column (400 × 3 mm I.D.) of Porasil A (60), 37–75 μm, in binary mobile phases composed of ethyl acetate and cyclohexane in different proportions. The values of the capacity ratios, $k'$, that are not given in the table can be calculated easily as the half of the corresponding $V_R'$ value ($V_m$ = 2.00 ml).

TABLE I

EXPERIMENTAL AND CALCULATED VALUES OF THE ELUTION CHARACTERISTICS OF THE N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOYLAMIDES IN ADSORPTION CHROMATOGRAPHY ON PORASIL A (ISOCRATIC ELUTION)

Column dimensions: 400 × 3 mm; $V_m$ = 2.00 ml. Adsorbent: Porasil A(60), 37–75 μm. Mobile phase: mixture of cyclohexane and ethyl acetate. Flow-rate: 38.5 ml/h. Temperature: 20–22°. Detection: photometric, 440 nm. Chromatographed compounds: 1 = dimethylamide; 2 = diethylamide; 3 = di-*n*-propylamide; 4 = di-*n*-butylamide derivative; *ca.* 8 μg each. $c$ = volume concentration of ethyl acetate; $x$ = mole fraction of ethyl acetate. The experimental values represent the arithmetic means from three experiments.

| $c$ | $x$ | Compound | $V'_R$ (ml) | | $w$ (ml) | | $N$ | | $R_s$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Calc. | Exptl. | Calc. | Exptl. | Average | Measured | Calc. | Exptl. |
| 1 | 1 | 1 | 4.11 | 3.87 | 2.09 | 1.81 | 137 | 168 | | |
| | | 2 | 1.11 | 1.40 | 1.06 | — | — | — | 1.90 | — |
| | | 3 | 0.51 | 0.76 | 0.86 | — | — | — | 0.62 | — |
| | | 4 | 0.33 | — | 0.80 | — | — | — | 0.22 | — |
| 0.7 | 0.719 | 1 | 7.98 | 7.88 | 3.41 | 3.33 | 137 | 141 | | |
| | | 2 | 2.15 | 2.43 | 1.42 | — | — | — | 2.41 | — |
| | | 3 | 0.98 | 1.27 | 1.02 | — | — | — | 0.96 | — |
| | | 4 | 0.65 | 0.92 | 0.91 | — | — | — | 0.34 | — |
| 0.6 | 0.622 | 1 | 10.63 | 10.22 | 4.32 | 4.04 | 137 | 146 | | |
| | | 2 | 2.86 | 3.00 | 1.66 | — | — | — | 2.60 | — |
| | | 3 | 1.31 | 1.56 | 1.13 | — | — | — | 1.11 | — |
| | | 4 | 0.86 | 1.02 | 0.98 | — | — | — | 0.43 | — |
| 0.5 | 0.523 | 1 | 14.93 | 12.82 | 5.79 | 4.74 | 137 | 156 | | |
| | | 2 | 4.02 | 4.13 | 2.06 | 2.30 | 137 | 114 | 2.78 | 2.46 |
| | | 3 | 1.84 | 2.04 | 1.31 | — | — | — | 1.29 | — |
| | | 4 | 1.21 | 1.43 | 1.10 | — | — | — | 0.52 | — |
| 0.4 | 0.422 | 1 | 22.61 | 22.96 | 8.41 | 8.21 | 137 | 148 | | |
| | | 2 | 6.09 | 6.18 | 2.76 | 3.07 | 137 | 114 | 2.96 | 2.97 |
| | | 3 | 2.78 | 2.97 | 1.63 | — | — | — | 1.51 | — |
| | | 4 | 1.83 | 1.85 | 1.31 | — | — | — | 0.65 | — |
| 0.3 | 0.320 | 1 | 38.60 | 39.77 | 13.87 | 13.57 | 137 | 152 | | |
| | | 2 | 10.39 | 10.67 | 4.23 | 4.39 | 137 | 133 | 3.12 | 3.24 |
| | | 3 | 4.75 | 4.99 | 2.31 | 2.79 | 137 | 100 | 1.72 | 1.58 |
| | | 4 | 3.12 | 3.20 | 1.75 | 1.98 | 137 | 110 | 0.80 | 0.75 |
| 0.2 | 0.215 | 1 | 82.06 | — | 28.73 | — | — | — | | |
| | | 2 | 22.10 | 23.25 | 8.24 | 8.66 | 137 | 136 | 3.24 | — |
| | | 3 | 10.10 | 10.22 | 4.14 | 3.72 | 137 | 173 | 1.94 | 2.10 |
| | | 4 | 6.64 | 6.47 | 2.95 | 3.04 | 137 | 124 | 0.98 | 1.11 |
| 0.1 | 0.109 | 1 | 297.87 | — | 102.48 | — | — | — | | |
| | | 2 | 80.21 | 97.94 | 28.09 | 31.12 | 137 | 165 | 3.33 | — |
| | | 3 | 36.65 | 45.55 | 13.21 | 15.66 | 137 | 148 | 2.11 | 2.24 |
| | | 4 | 24.11 | 28.96 | 8.92 | 9.08 | 137 | 186 | 1.13 | 1.29 |

The logarithms of the $V'_R$ values given in Table I are plotted *versus* the logarithms of the mole fraction ($x$) of ethyl acetate as the more efficient eluting component in the binary mobile phase in Fig. 2. The relationships are linear for all of the compounds studied, which indicates the validity of eqn. 1 in the system used. Some more

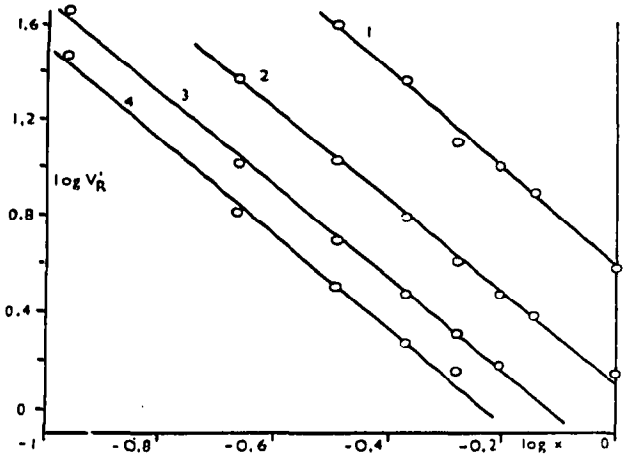


Fig. 2. Relationship between the mole fraction of ethyl acetate ($x$) in a binary mobile phase (ethyl acetate–cyclohexane) and the retention volumes of N,N-dimethyl-*p*-aminobenzeneazobenzoylamides in chromatography on Porasil A (37–75 $\mu$m). 1 = Dimethylamide; 2 = diethylamide; 3 = di-*n*-propylamide; 4 = di-*n*-butylamide derivative. Column dimensions: 400 × 3 mm; $V_m$ = 2.00 ml. Flow-rate: 38.5 ml/h. Detection: photometric, 440 nm.

significant deviations of the experimental values from the linear plot can be observed at values of log $V_R'$ less than 0.18 ($V_R' < 1.5$ ml). This is not surprising, because a small experimental error in the determination of retention volumes or $V_m$ would introduce a considerable deviation on the logarithmic scale (log $V_R'$) in this region of small retention volumes.

The constants $n$ and $k_0'$ in eqn. 1 calculated for the four amides from the respective $V_R'$ and $x$ values (Fig. 2, Table I) are given in Table II (Adsorbent II[a]). The

TABLE II

EXPERIMENTAL VALUES OF THE PARAMETERS $n$ AND $k_0'$ IN EQN. 1 FOR N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOYLAMIDES ON SILICA IN MOBILE PHASE ETHYL ACETATE-CYCLOHEXANE

The values were evaluated from the plots of log $k'$ against logarithms of the mole fraction of ethyl acetate ([a]) or the logarithms of the volume concentration of ethyl acetate ([b]). Chromatographed compounds: 1 = dimethylamide; 2 = diethylamide; 3 = di-*n*-propylamide; 4 = di-*n*-butylamide derivative. Adsorbents: I = Kieselgel; II = Porasil A (60), 37–75 $\mu$m; III = Porasil A (60), 37–75 $\mu$m as II, but deactivated by passing 1 ml of 94% aqueous ethanol through the column. Experimental conditions as in Table I.

| *Compound* | *Adsorbent* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *I* | | *II* | | *II* | | *III* | |
| | $n^a$ | $k_0'^a$ | $n^a$ | $k_0'^a$ | $n^b$ | $k_0'^b$ | $n^a$ | $k_0'^a$ |
| 1 | 2.25 | 1.93 | 2.04 | 1.96 | 1.93 | 1.94 | 1.78 | 3.05 |
| 2 | 2.23 | 0.52 | 1.84 | 0.65 | 1.76 | 0.65 | 1.73 | 1.11 |
| 3 | 2.22 | 0.23 | 1.89 | 0.31 | 1.68 | 0.33 | 1.72 | 0.55 |
| 4 | 1.96 | 0.21 | 1.85 | 0.20 | 1.68 | 0.24 | 1.67 | 0.38 |
| Arithmetic mean | 2.16 | — | 1.91 | — | 1.76 | — | 1.72 | — |

values of the exponent $n$ are close each to the other for all of the amides studied, with a maximum difference between the individual amides of about 10%. In Part I[1], the exponent $n$ in adsorption chromatography was defined as

$$n = \frac{A_s}{n_b} \tag{12}$$

where $A_s$ is the molecular area of an adsorbed sample molecule on the adsorbent surface and $n_b$ is the effective molecular area of an adsorbed solvent molecule of the stronger eluting component in the binary mobile phase (ethyl acetate). The values of $n_b$ have been tabulated for a number of solvents[7]; $n_b$, as a property of the solvents used, is constant for all of the binary mobile phases that contain the same more polar component. Therefore, any differences in the values of $n$ between the individual sample compounds can be attributed to the differences in the $A_s$ values. A larger value of $n$ thus indicates that the sorbed sample molecules occupy a larger area of the adsorbent surface. The agreement between the values of $n$ observed in the series of homologous N,N-dimethyl-*p*-aminobenzeneazobenzoylamides shows that the molecules of all of the homologues occupy approximately the same area of the adsorbent surface. This is possible if the alkyl groups in the sorbed molecules of an amide are oriented towards the outer solution and are not in direct contact with the surface of silica. These considerations are in agreement with the published data on the adsorption of aromatic molecules that contain alkyl chains, according to which the whole of the areas of the aromatic parts of molecules and polar groups are adsorbed on the surface of silica and the alkyl chains are partly desorbed. This desorption increases with increase in the number of carbon atoms in the alkyl chain[7].

Table II also gives the values of the constants $n$ and $k'_0$ found in another set of experiments carried out on the same column packed with Porasil A, after all the other experiments with isocratic and gradient elution had been finished. In these experiments, the activity of Porasil was decreased by passing 1 ml of 84% ethanol through the column (Adsorbent III). Then, the column was re-equilibrated with ethyl acetate and cyclohexane and the experiments were carried out and evaluated in the same way as the experiments with isocratic elution on the more active Porasil A (Adsorbent II). As expected, the $k'_0$ values on deactivated Porasil are larger than those on the more active adsorbent, while the differences between the values of $n$ in the two sets of experiments are not very significant —the area of the adsorbent occupied by an adsorbed sample molecule does not change with a decrease in adsorbent activity.

The plot of $\log k'$ for the four amides *versus* the mole fraction of ethyl acetate in the binary mobile phase (cyclohexane–ethyl acetate) on a column of the same dimensions (400 × 3 mm) packed with Kieselgel (<50 μm) is also linear. The respective $n$ and $k'_0$ values are included in Table II. The differences between the values of $n$ measured on Kieselgel (Adsorbent I) and the values for the same amides on Porasil A do not exceed the experimental error. This agreement indicates that the area of the adsorbed molecule of an amide, $A_s$, is approximately the same on the surface of both adsorbents, which have the same average pore diameter (60 Å). The $k'_0$ values are controlled by the porosity and specific surface of the adsorbent and its activity, and these properties could be different for the two adsorbents.

The gradient-generating pump is able to mix liquids in volume ratios, not in mole ratios. As the concentration $c$ in eqns. 1, 2 and 4–11 is expressed as a mole fraction,

TABLE III

COMPARISON OF THE $n$ AND $k'_0$ VALUES IN EQN. 1 FOR N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOYLAMIDES ON PORASIL A (60), 37–75 $\mu$m, IN THE MOBILE PHASE CYCLOHEXANE–ETHYL ACETATE

The values of $n$ and $k'_0$ were determined from the coefficients of the regression lines for the experimental log $V'_R$ values as a function (1) of logarithms of the volume concentration of ethyl acetate in the mobile phase (isocratic elution) (I), and (2) of logarithms of the parameter $B$ in gradient elution at $c_0 = A = 0$ for different $\varkappa$ values: $\varkappa = 0.5$ (II); $\varkappa = 1.0$ (III); $\varkappa = 2.0$ (IV) and $\varkappa = 4.0$ (V). Chromatographed compounds: 1 = dimethylamide; 2 = diethylamide; 3 = di-*n*-propylamide; 4 = di-*n*-butylamide derivative. Operating conditions are specified in Tables I and IV–VIII. $\Delta n$ relates to the deviation (%) from the average value of $n = 1.860$, which is common to all four amides studied. $\Delta k'_0$ expresses the deviations (%) from the arithmetic means for each compound.

| *Experimental conditions* | *Compound* | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *1* | | | | *2* | | | | *3* | | | | *4* | | | |
| | $n$ | $\Delta n$ | $k'_0$ | $\Delta k'_0$ | $n$ | $\Delta n$ | $k'_0$ | $\Delta k'_0$ | $n$ | $\Delta n$ | $k'_0$ | $\Delta k'_0$ | $n$ | $\Delta n$ | $k'_0$ | $\Delta k'_0$ |
| I | 1.93 | + 3.8 | 1.94 | − 5.6 | 1.76 | − 5.4 | 0.65 | +16.7 | 1.68 | − 9.7 | 0.330 | +30.4 | 1.68 | − 9.7 | 0.242 | +45.4 |
| II | 1.84 | − 1.1 | 1.94 | − 5.6 | 1.94 | + 4.3 | 0.49 | −11.8 | 1.95 | + 4.8 | 0.220 | −13.0 | 1.87 | + 0.5 | 0.164 | − 1.4 |
| III | 1.45 | −22.0 | 2.59 | +25.8 | 1.68 | − 9.7 | 0.69 | +24.6 | 1.71 | − 8.1 | 0.325 | +28.5 | 1.75 | − 5.9 | 0.202 | +21.4 |
| IV | 1.92 | + 3.2 | 1.75 | −15.1 | 2.20 | +18.3 | 0.36 | −35.7 | 2.33 | +25.3 | 0.127 | −49.8 | 2.41 | +32.8 | 0.068 | −59.1 |
| V | 1.70 | − 8.6 | 2.07 | + 0.6 | 1.74 | − 6.5 | 0.59 | + 6.2 | 1.80 | − 3.2 | 0.263 | + 4.0 | 1.85 | − 0.5 | 0.156 | − 6.3 |
| Arithmetic mean | 1.768 | − 4.9 | 2.056 | − | 1.864 | + 0.2 | 0.554 | − | 1.894 | + 1.8 | 0.253 | − | 1.912 | + 2.8 | 0.166 | − |

it is necessary to compensate for the difference between the two concentration expressions by introducing an appropriate mathematical correction of the gradient function. The ratios of the density and molecular weight ($100 \cdot d/M$) for cyclohexane and ethyl acetate are similar and close to unity (0.93 for cyclohexane and 1.02 for ethyl acetate)[7], and consequently the mole fraction does not differ much from the volume concentration of ethyl acetate in cyclohexane. These differences do not exceed 10% in the concentration range 10–100% of ethyl acetate (Table I). Therefore, it was expected that the volume concentrations could be used instead of the mole fractions for the construction of the gradient function (concentration of ethyl acetate in the mobile phase *versus* the volume of the eluate or time) without introducing serious errors. This assumption was tested experimentally.

Values of log $V_R'$ for the four N,N-dimethyl-*p*-aminobenzeneazobenzoylamides are plotted in Fig. 3 *versus* the logarithms of the volume concentration of ethyl acetate in the binary mobile phase. The lines represent theoretical relationships based on the av-

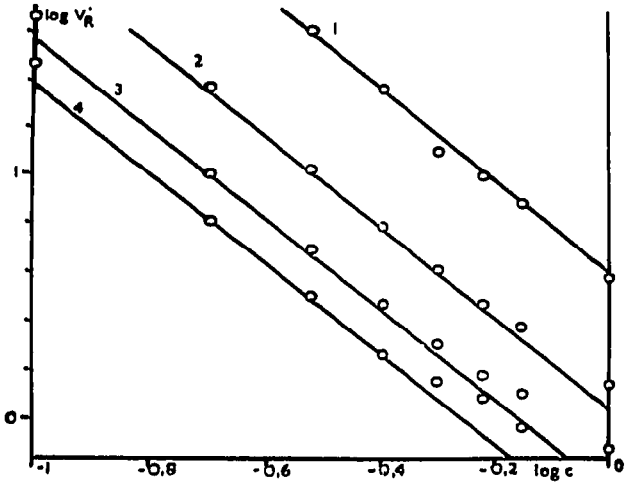


Fig. 3. Relationship between the volume concentration of ethyl acetate ($c$) in a binary mobile phase (ethyl acetate–cyclohexane) and the retention volumes of N,N-dimethyl-*p*-aminobenzeneazobenzoylamides in chromatography on Porasil A (37–75 μm). 1 = Dimethylamide; 2 = diethylamide; 3 = di-*n*-propylamide; 4 = di-*n*-butylamide derivative. Operating conditions as in Fig. 2.

erage values of $n$ and $k_0'$, which were determined not only from the experimental data in isocratic elution chromatography (points in Fig. 3), but also from experiments with gradient elution at four different gradient functions. These values of $n$ and $k_0'$ are given in Table III and the calculation is based on the average values of $k_0'$ and $n$ ($n = 1.860$). The lines therefore do not represent regression lines.

Comparison of the relationships log $V_R' = f(\log x)$ and log $V_R' = f(\log c)$ in Figs. 2 and 3 ($x$ = mole fraction; $c$ = volume concentration of ethyl acetate in cyclohexane) shows an equally good linearity in both instances. The deviations between the experimental values and the theoretical plots of log $V_R'$ *versus* log $c$ exceed those found for the relationship based on the mole fraction only in the mobile phase containing 10% (or less) of ethyl acetate, where the difference between the mole and volume concentrations is most distinct (about 9%). The mobile phase with this composition has a low polarity and is a weak eluting agent (large $k'$ values) and therefore the

deviations observed can be assumed to have only minor significance in gradient elution chromatography.

The values of the exponent $n$ evaluated from the relationship $\log V_R' = f(\log c)$ are only about 5–10% lower than those found from the relationship between $\log V_R'$ and $\log x$. The $k_0'$ values are lower by 0.004–0.044 in the first instance (Table II). These results encouraged us to express the concentration of ethyl acetate in the binary mobile phase as a volume concentration for the construction of gradient functions (eqn. 3) to be used in gradient elution chromatography in cyclohexane–ethyl acetate. Thus, in the following discussion, the term concentration will refer to the volume concentration of ethyl acetate in the mobile phase.

Table I compares the experimental values of the retention volume ($V_R'$), peak width ($w$), plate number ($N$) and resolution ($R_s$) with the values calculated from the average parameters $k_0'$ and $n$ (Table II) using eqns. 4–6. The differences between the experimental and calculated values of retention volume do not exceed 0.4 ml when $V_R'$ is less than 25 ml (except for one value). Somewhat larger deviations in the mobile phase containing 10% of ethyl acetate could be caused by the distinct difference between the volume and mole concentrations of ethyl acetate, *i.e.*, about 9%. The agreement between the experimental and calculated values of $w$ and $R_s$ is also satisfactory. In addition, the experimental plate numbers are compared with the average value ($N = 137$).

Good agreement between the experimental and calculated data in Table I indicates that eqns. 4–6 derived in Part I[1] are suitable for calculation of the elution characteristics ($V_R'$, $w$ and $R_s$) in the isocratic elution adsorption chromatography of N,N-dimethyl-*p*-aminobenzeneazobenzoylamides on silica in binary solvent systems composed of cyclohexane and ethyl acetate, even if the concentration of ethyl acetate is expressed as the volume concentration.

*Gradient elution*

In gradient elution experiments, the validity of eqns. 8–11 derived in Part II[2] was tested experimentally, using the same system as in isocratic elution chromatography. The composition of the binary mobile phase (concentration of ethyl acetate in cyclohexane) was changed continuously during the elution according to the gradient function (eqn. 3). The concentration of ethyl acetate was expressed as a volume concentration in this equation. Because the flow-rate of the mobile phase was not influenced by its composition, any eventual volume contractions caused by mixing the two components of the mobile phase were assumed to be negligible.

Fig. 4 shows the graphs of the concentration gradient functions tested. Fig. 4, I–V, shows twenty simple gradient functions based on eqn. 3 with different values of the parameters $\varkappa$, $A$ and $B$ ($c_0$, $c_k$) represented in curves 1–20. In addition, stepwise elution (curve 22 in Fig. 4, VI) and combined two-step elution, isocratic in the first step with a linear gradient in the second step (curve 21 in Fig. 4, VI), were also studied and will be discussed in Part IV of this series.

The influence of the parameters $\varkappa$ and $B$ ($c_k$) on the chromatographic behaviour of the four amides studied was tested at a constant value of $A = c_0 = 0$ ($V_y = 38.5$ ml) for four values (0.5, 1, 2 and 4) of the parameter $\varkappa$, which controls the shape (curvature) of the gradient function. For each value of $\varkappa$, four gradient functions were studied, differing in the value of $B$ ($c_k$). Fig. 4, I, shows curves 1–4 for $\varkappa = 0.5$;

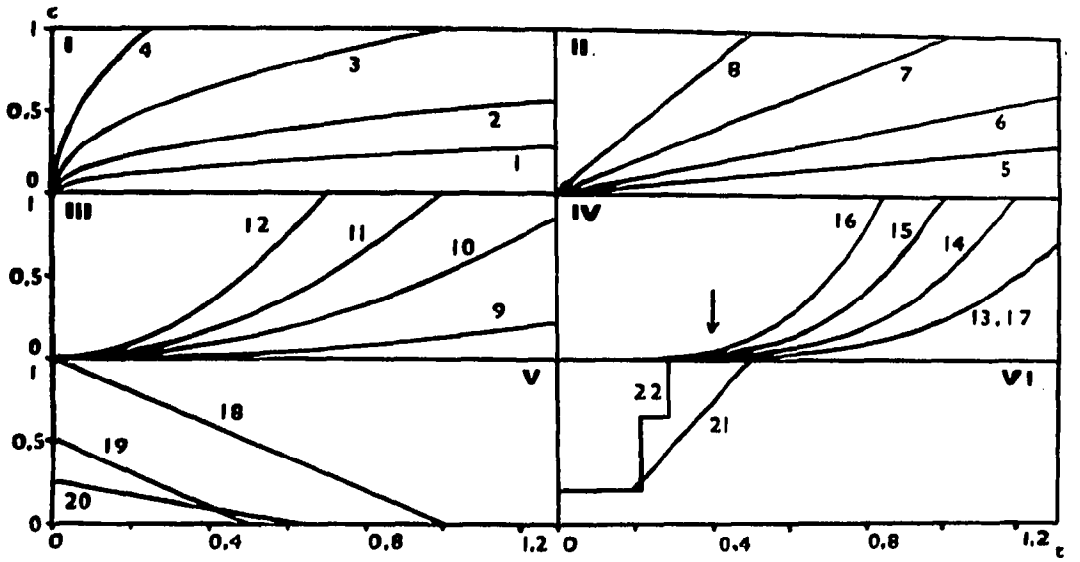


Fig. 4. Survey of gradient functions (volume concentration of ethyl acetate in cyclohexane ($c$) *versus* time ($t$)) used in gradient elution experiments. Curves 1–16 and 18–20 follow eqn. 3. Curve 17 is identical with curve 13, except that the elution was started not from the beginning ($t = 0$) but from the point marked by the arrow (corresponding to a volume of the eluate of $V = 15.40$ ml). The experiments with stepwise and combined two-step isocratic gradient elution (curves 21 and 22) will be discussed in Part IV of this series. The values of the parameters of gradient elution were as follows. $\varkappa = 0.5$ (I; curves 1–4); $\varkappa = 1$ (II; V; curves 5–8, 18–20); $\varkappa = 2$ (III; curves 9–12); $\varkappa = 4$ (IV; curves 13–17). $A = c_0 = 0$ (I–IV; curves 1–17); $c_0 = 1$ (V; curve 18); $c_0 = 0.5$ (V; curve 19); $c_0 = 0.25$ (V; curve 20). $c_k = 0$ (V; curves 18–20); $c_k = 0.125$ (III; curve 9); $c_k = 0.25$ (curves 1, 5, 13 and 17); $c_k = 0.5$ (curves 2, 6, 10 and 14); $c_k = 1$ (curves 3, 7, 11 and 15); $c_k = 2$ (curves 4, 8, 12 and 16). $V_y = 38.5$ ml (curves 1–18); $V_y = 19.25$ ml (curve 19); $V_y = 24.06$ ml (curve 20). The values of the parameter $B$ (eqn. 3b) are given in Tables IV–VIII.

Fig. 4, II, shows curves 5–8 for $\varkappa = 1.0$; Fig. 4, III, shows curves 9–12 for $\varkappa = 2.0$; and Fig. 4, IV, shows curves 13–17 for $\varkappa = 4$.

Figs. 5 and 6 show the separations achieved with different $\varkappa$ and $B$ ($c_k$) values in gradient elution chromatography. Fig. 5 illustrates this separation obtained for different $B$ ($c_k$) values at constant values of $\varkappa$ and $A$ ($\varkappa = 1$; $A = c_0 = 0$), *i.e.*, for linear gradient functions with different slopes. The chromatograms obtained for different $\varkappa$ values at constant $A$ and $B$ ($c_0$ and $c_k$), *i.e.*, for different shapes (curvatures) of the gradient function at a constant total concentration change during the gradient elution, are shown in Fig. 6. For comparison, Fig. 7 shows chromatograms obtained at four different concentrations of ethyl acetate in the mobile phase (70, 50, 30 and 20%) in isocratic elution chromatography.

Fig. 7 shows an example of a situation referred to as the "general elution problem", which is connected with isocratic elution. When a sample contains several components with widely varying $k'$ values, it is impossible to adjust simultaneously all these values to give a good separation by a choice of one composition of the mobile phase. In such an instance, some compounds may be well separated, but other components have $k'$ values that are too small and are eluted early with poor or no separa-

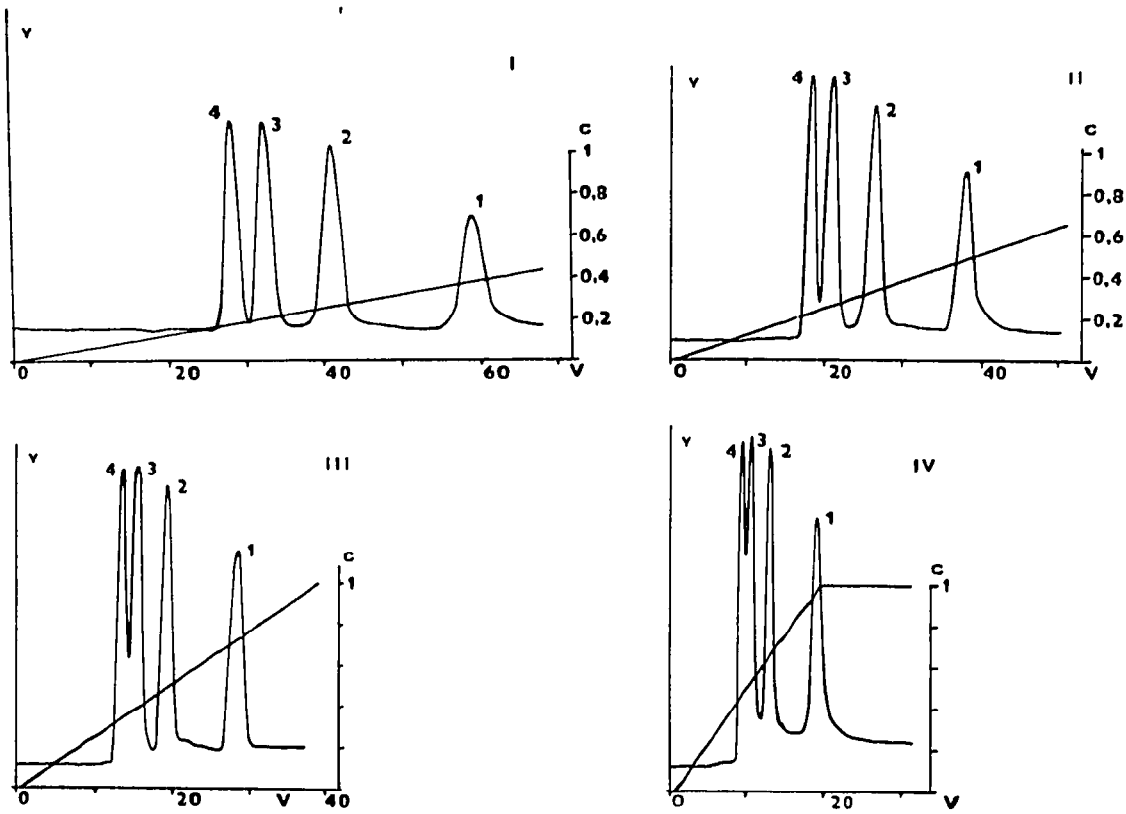


Fig. 5. Gradient elution chromatographic separation of a mixture of the dimethylamide (1), diethylamide (2), di-*n*-propylamide (3), and di-*n*-butylamide (4) derivatives on a column (400 × 3 mm) packed with Porasil A ($V_m$ = 2.00 ml) using ethyl acetate–cyclohexane mixtures as mobile phase. Linear gradient function; different slope of the gradient; $\varkappa = 1$, $A = c_0 = 0$; $V_y$ = 38.5 ml. I, $c_k$ = 0.25; $B$ = 0.006494 (curve 5 in Fig. 4). II, $c_k$ = 0.5; $B$ = 0.012987 (curve 6 in Fig. 4). III, $c_k$ = 1.0; $B$ = 0.025974 (curve 7 in Fig. 4). IV, $c_k$ = 2.0; $B$ = 0.051948 (curve 8 in Fig. 4). $V$ = volume of the eluate (ml); $y$ = detector response. The figure also shows the dependence of the concentration ($c$) (volume concentration) of ethyl acetate on the inlet of the column on $V$. Other operating conditions as in Fig. 2.

tion and some others may have $k'$ values that are too large and are eluted only after a long time as wide bands that often cannot be detected in the eluate.

In 70% ethyl acetate, the di-*n*-propyl- and di-*n*-butylamide derivatives are eluted together in one band and the separation of the diethylamide from the di-*n*-propylamide derivative is only partial. All the compounds are eluted in about 11 ml of the eluate. The separation of the diethylamide from the di-*n*-propylamide derivative is almost quantitative in 50% ethyl acetate. In this case, a slight splitting of the first peak is observed, corresponding to the di-*n*-propyl- and di-*n*-butylamide derivatives. The separation requires about 20 ml of the mobile phase. In 30% ethyl acetate, the elution of the four amides is accomplished with about 50 ml of the mobile phase and the separation of the diethyl- and di-*n*-propylamide derivatives is complete. The di-*n*-butylamide derivative is only partly separated from the di-*n*-propylamide derivative in this mobile phase, while a satisfactory separation of these two compounds is

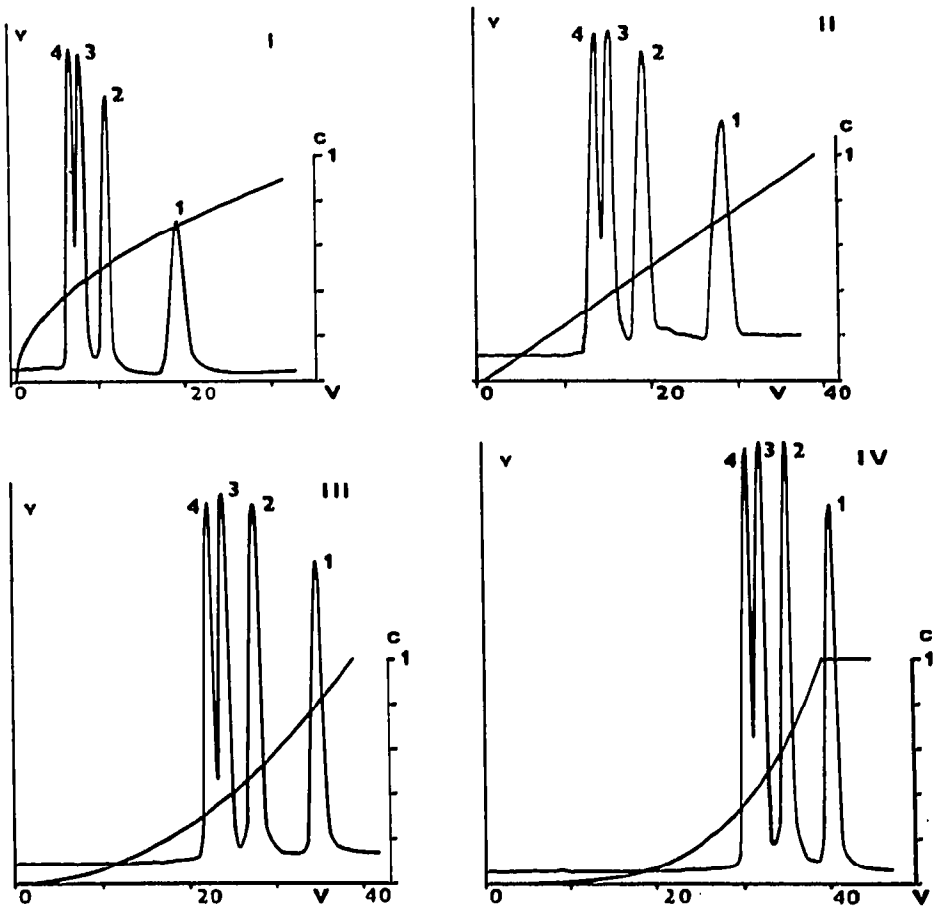


Fig. 6. Gradient elution chromatographic separation of a mixture of the dimethylamide (1), diethylamide (2), di-*n*-propylamide (3) and di-*n*-butylamide (4) derivatives on a column (400 × 3 mm) packed with Porasil A ($V_m$ = 2.00 ml) using ethyl acetate–cyclohexane mixtures as mobile phase. Constant total concentration change of ethyl acetate (gradient slope), different shape (curvature) of the gradient; $A = c_0 = 0$; $c_k = 1$; $V_y = 38.5$; $B = 0.025974$. I, $\varkappa = 0.5$ (curve 3 in Fig. 4); II, $\varkappa = 1.0$ (curve 7 in Fig. 4). III, $\varkappa = 2.0$ (curve 11 in Fig. 4). IV, $\varkappa = 4.0$ (curve 15 in Fig. 4). $V$ = volume of the eluate (ml); $y$ = detector response. The figure also shows the dependence of the concentration ($c$) (volume concentration) of ethyl acetate on the inlet of the column on $V$. Other operating conditions as in Fig. 2.

achieved in 20% ethyl acetate. In this case, however, about 32 ml of the mobile phase are required for the elution of the first three components and the elution of dimethylamide is very slow.

Comparison of Fig. 7 with Figs. 5 and 6 clearly demonstrates the capability of gradient elution chromatography to achieve a separation comparable with or even better than that in isocratic elution chromatography, using a considerably smaller volume of the mobile phase. Figs. 5 and 6 also show the influence of the parameters $B$ ($c_k$) and $\varkappa$ on the separation in gradient elution chromatography controlled by eqn. 3. A decrease in $B$ (or $c_k$ at $c_0$ = constant = 0) at a constant $\varkappa$ value is connected with a simultaneous improvement in separation and an increase in retention volumes of

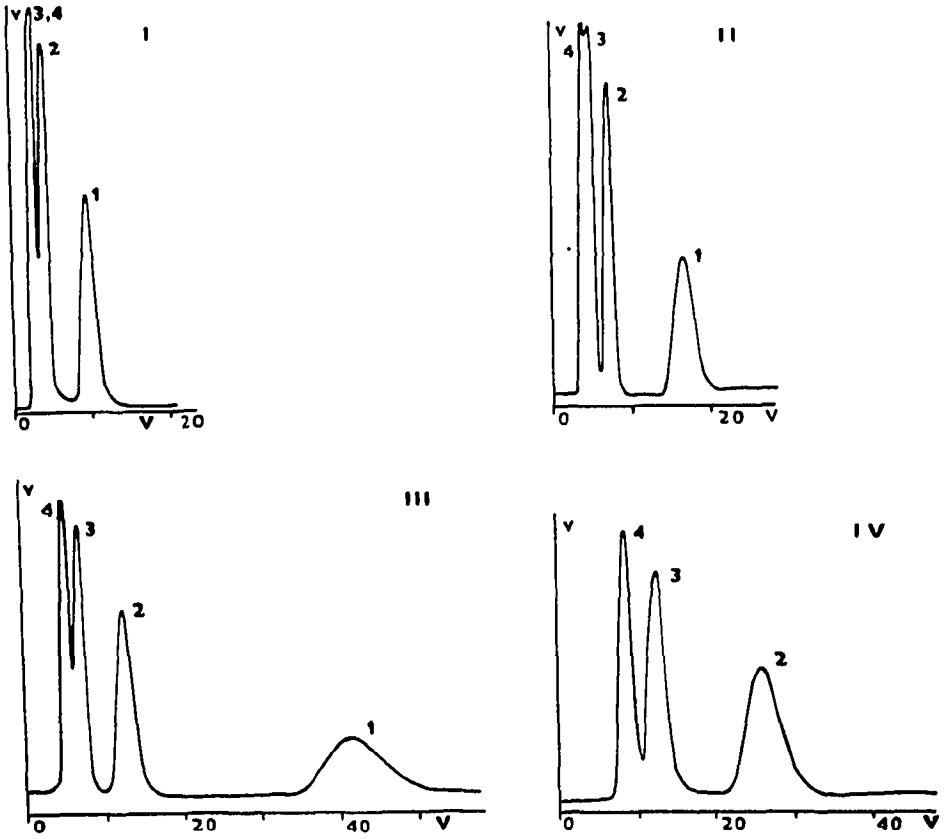


Fig. 7. Chromatographic separation of a mixture of the dimethylamide (1), diethylamide (2), di-*n*-propylamide (3) and di-*n*-butylamide (4) derivatives on a column (400 × 3 mm) packed with Porasil A ($V_m$ = 2.00 ml) using ethyl acetate–cyclohexane mixtures of constant composition as mobile phase. Volume concentration of ethyl acetate in the mobile phase: I, $c$ = 0.7; II, $c$ = 0.5; III, $c$ = 0.3; IV, $c$ = 0.2. $V$ = volume of the eluate (ml); $y$ = detector response. Other operating conditions as in Fig. 2.

the sample compounds, as shown in Fig. 5 for $\varkappa = 1$. Fig. 6 shows that the influence of the parameter $\varkappa$ (for $\varkappa$ in the range 0.5–4.0) on the quality of separation is of only minor importance. The greatest differences are observed in the separation of dimethylamide and diethylamide. An increase in $\varkappa$ decreases the distance between the elution bands of these two compounds and the distances between all of the neighbouring elution bands become spaced more regularly. The volume of the mobile phase required for the separation and the time of analysis, however, increase with increase in $\varkappa$. High $\varkappa$ values cause the concentration of the efficient eluting component in the mobile phase to differ only negligibly from the initial value ($c_0$), which is zero for a considerable time in the experiments under discussion. This means that a large part of the gradient remains useless for the separation, as the $k'$ values of the amides are very large in pure cyclohexane and this solution is a very inefficient eluting agent. This inefficient part of the gradient programme corresponds to *ca.* 3 ml for $\varkappa = 2$ (about 8% of the total separation time) and to *ca.* 10 ml for $\varkappa = 4$ (which corresponds to about 24% of the

total separation time). If the elution is started from that point of the gradient function at which the concentration of ethyl acetate begins to differ significantly from $c_0 = 0$, both the widths of the peaks and the distances between the peak maxima are the same as in elution utilizing the whole gradient function as given by eqn. 3, and only the retention volumes are decreased by a value corresponding to the omitted part of the gradient. This is illustrated by the results of the gradient elution according to curves 13 and 17 in Fig. 4 ($\varkappa = 4$; $c_k = 0.25$), which are given later in Table VII.

The gradient elution according to the curve 17 was different from the elution employing curve 13 (controlled by eqn. 3), in that it was started at the point corresponding to a volume of the eluate of $V = 15.40$ ml. This value was subtracted from the retention volumes, $V'_{R(g)}$, calculated according to eqn. 8 prior to the substitution into eqns. 10 and 11 for the calculation of the other elution characteristics ($R_{s(g)}$ and $N_{(g)}$). The values calculated in this way are in equally good agreement with experiment as the values found for curve 13 controlled by eqn. 3.

Hence, the resolution and time of analysis in gradient elution chromatography are controlled mainly by the slope of the gradient and the initial concentration of the more efficient eluting agent in the binary mobile phase (expressed by $c_0$, $c_k$ and $B$). The shape (curvature) of the gradient function (at constant $c_0$ and $c_k$ values) influences the position of the elution bands in the chromatogram to a certain extent and is important for the time of analysis (total volume of the mobile phase required for separation).

The validity of the equation for the retention volume in gradient elution chromatography (eqn. 8) was tested by comparing the experimental and theoretical functional dependence of the retention volume on $B$. If $c_0 = 0$ is substituted for $A$ into eqn. 8 and $\varkappa$ is kept constant, the relationship between $V'_{R(g)}$ and $B$ can be written as

$$\log V'_{R(g)} = \frac{1}{\varkappa \cdot n + 1} \cdot \log [(\varkappa \cdot n + 1) \cdot k'_0 \cdot V_m] - \frac{\varkappa \cdot n}{\varkappa \cdot n + 1} \cdot \log B \qquad (13)$$

*i.e.*

$$\log V'_{R(g)} = a + b \cdot \log B \qquad (13a)$$

This is the equation of a line with a slope of $-\varkappa \cdot n/(\varkappa \cdot n + 1)$. Fig. 8 shows plots of the experimental (points) and theoretical (lines) functions of $\log V'_{R(g)}$ of the four amides studied *versus* $\log B$ at four values of $\varkappa$ (0.5, 1.0, 2.0 and 4.0) corresponding to the gradient functions represented by curves 1–16 in Fig. 4.

All the experimental points adhere closely to the theoretical lines, which are not the regression lines but represent the plots of the relationships calculated from the average values of $k'_0$ and $n$ from Table III (similarly to the lines in Fig. 3).

It is possible to determine the parameters $n$ and $k'_0$ from the constants $a$ and $b$ of eqn. 13a representing the parameters of the regression lines for the experimental points:

$$n = -\frac{b}{\varkappa \cdot (b + 1)} \qquad (14)$$

$$k'_0 = \frac{1}{(\varkappa \cdot n + 1) \cdot V_m} \cdot 10^{a \cdot (\varkappa \cdot n + 1)} \qquad (15)$$

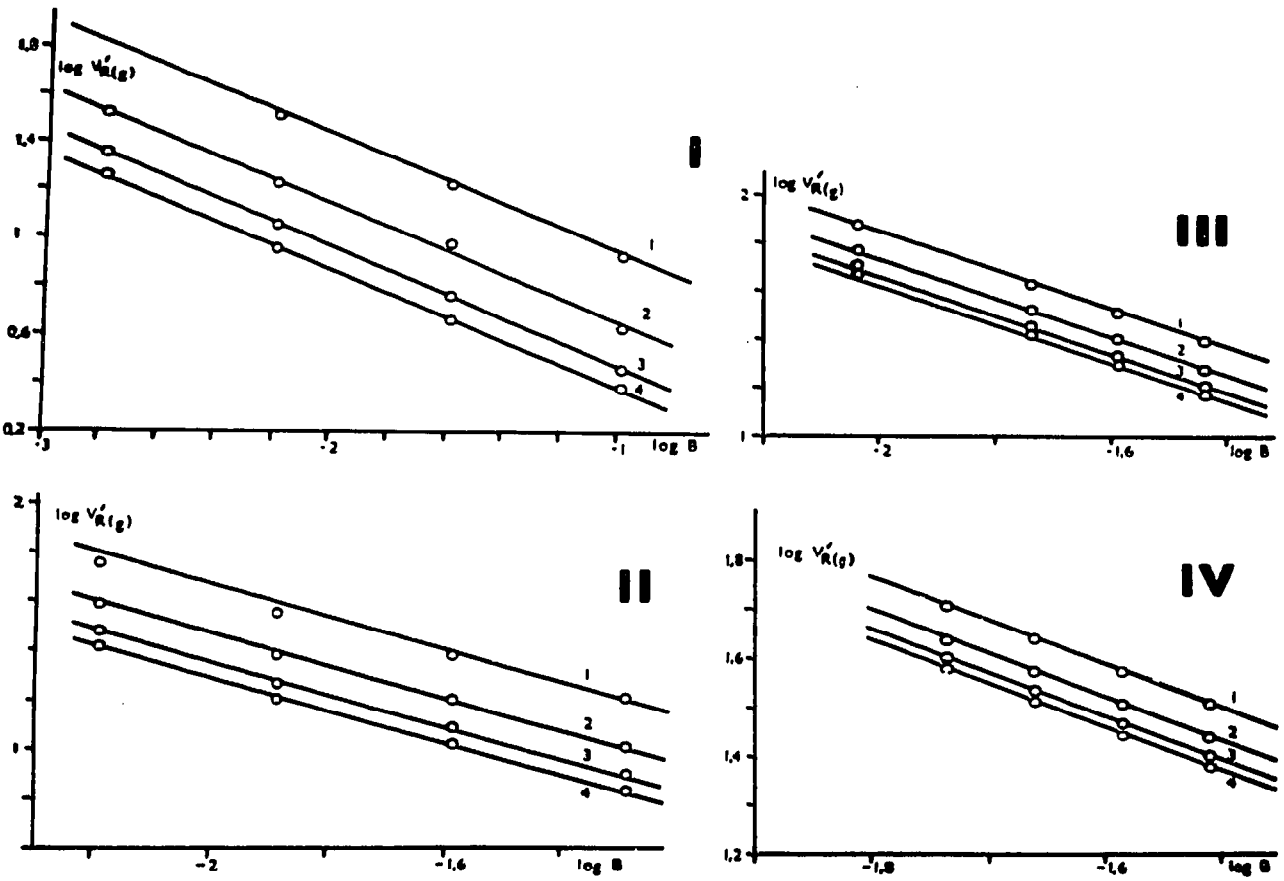


Fig. 8. Relationship between the retention volumes, $V'_{(g)}$ (ml), of N,N-dimethyl-*p*-aminobenzeneazobenzoylamides and the parameter $B$ in gradient elution chromatography on Porasil A (37–75 $\mu$m). Parameters of the gradient elution: $A = c_0 = 0$; $V_y = 38.5$ ml; I, $\varkappa = 0.5$; II, $\varkappa = 1.0$; III, $\varkappa = 2.0$; IV, $\varkappa = 4.0$. 1 = Dimethylamide; 2 = diethylamide; 3 = di-*n*-propylamide; 4 = di-*n*-butylamide derivative. Column dimensions: 400 × 3 mm; $V_m = 2.00$ ml. Flow-rate: 38.5 ml/h. Detection: photometric, 440 nm. The points represent the experimental values and the lines are not the regression lines, but theoretical relationships calculated from the average values of $n$ and $k'_0$ (Table III).

The values of these constants calculated at four different $\varkappa$ values are compared in Table III with the constants $k'_0$ and $n$ determined from the experiments with isocratic elution. Table III also gives the average constants calculated as the arithmetic means of the values found in all of the experiments with isocratic (I) and gradient elution (II–V) chromatography. The theoretical lines in Figs. 3 and 8 were constructed on the basis of these average values, which were also utilized for calculations of the theoretical values of elution characteristics (retention volume, peak width, plate number and resolution). These values are compared with the experimental values in Table I for isocratic and in Tables IV–VIII for gradient elution chromatography. One common average value of $n = 1.860$ was taken into consideration for all of the four amides studied.

The agreement between the values of $n$ evaluated in gradient elution chroma-

TABLE IV

EXPERIMENTAL AND CALCULATED VALUES OF THE ELUTION CHARACTERISTICS OF THE N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOYLAMIDES IN ADSORPTION CHROMATOGRAPHY ON PORASIL A

Gradient elution, $\varkappa = 0.5$; $A = c_0 = 0$; $V_y = 38.5$ ml. Column dimensions: 400 × 3 mm; $V_m = 2.00$ ml. Adsorbent: Porasil A (60), 37–75 $\mu$m. Flow-rate of mobile phase (ethyl acetate–cyclohexane): 38.5 ml/h. Temperature: 20–22°. Detection: photometric, 440 nm. Chromatographed compounds: 1 = dimethylamide; 2 = diethylamide; 3 = di-*n*-propylamide; 4 = di-*n*-butylamide derivative; *ca.* 8 $\mu$g each. The numbers of the experiments agree with the numbers of the curves in Fig. 4. The experimental values represent the arithmetic means from three experiments.

| *Experiment No.* | $c_k$ | $B$ | *Compound* | $V'_{R(g)}$ *(ml)* | | $w_{(g)}$ *(ml)* | | $N_{(g)}$ | | $R_{s(g)}$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* |
| 1 | 0.25 | 0.0016234 | 1 | 64.61 | — | 12.12 | — | 493 | — | 3.43 | — |
| | | | 2 | 32.74 | 32.88 | 6.48 | 5.77 | 478 | 585 | 1.89 | 2.14 |
| | | | 3 | 22.33 | 22.26 | 4.55 | 4.13 | 483 | 552 | 1.14 | 1.16 |
| | | | 4 | 17.56 | 17.83 | 3.79 | 3.46 | 456 | 526 | | |
| 2 | 0.5 | 0.0064935 | 1 | 33.13 | 31.69 | 6.55 | 5.13 | 478 | 690 | 3.20 | 3.65 |
| | | | 2 | 16.79 | 16.52 | 3.66 | 3.17 | 452 | 546 | 1.77 | 2.00 |
| | | | 3 | 11.19 | 11.06 | 2.66 | 2.27 | 434 | 530 | 0.89 | 0.99 |
| | | | 4 | 9.00 | 8.88 | 2.28 | 2.11 | 419 | 425 | | |
| 3 | 1.0 | 0.025974 | 1 | 16.99 | 16.39 | 3.69 | 3.17 | 454 | 538 | 2.84 | 3.07 |
| | | | 2 | 8.61 | 8.46 | 2.21 | 1.98 | 417 | 447 | 1.47 | 1.54 |
| | | | 3 | 5.74 | 5.64 | 1.70 | 1.66 | 392 | 339 | 0.70 | 0.74 |
| | | | 4 | 4.62 | 4.52 | 1.50 | 1.37 | 378 | 362 | | |
| 4 | 2.0 | 0.103896 | 1 | 8.71 | 8.40 | 2.23 | 2.34 | 417 | 316 | 2.33 | 2.18 |
| | | | 2 | 4.41 | 4.23 | 1.46 | 1.47 | 376 | 287 | 1.11 | — |
| | | | 3 | 2.94 | 2.85 | 1.20 | — | 350 | — | 0.50 | — |
| | | | 4 | 2.37 | 2.40 | 1.10 | — | 336 | — | | |

tography and the average value of $n = 1.860$ is satisfactory; the deviations exceed 10% only for four of the sixteen values measured. The relative deviations of $k'_0$ from the average values are larger, but for only five of the sixteen values measured do they exceed 25% (Table III). The precision of the determination of $n$ from the gradient elution data is comparable with the determination from experiments with isocratic elution. The estimation of $k'_0$ may be less confident in the first case owing to the errors connected with the substitution of the experimental values of $n$ into the complex eqn. 15. The series of the experiments at $\varkappa = 2$ was subject to a larger error than the other series, where the results were satisfactory.

The agreement between the experimental and calculated values of the retention volumes in gradient elution chromatography (eqn. 8) is good (Tables IV–VII); only one experimental value of the 68 tested shows a deviation from the calculated value larger than 10%. Also, the experimental peak widths agree with the calculated values (eqn. 9) and only five of the 65 experimental values tested deviate from the calculated values by more than 20%. These larger deviations occur in gradient elution with $\varkappa \leqslant 2$ and $c_k \leqslant 0.5$ for the most strongly adsorbed compound (the dimethylamide derivative). In general, the calculation according to the simplified eqn. 9 yields higher values of $w_{(g)}$ than the experimental values. In most instances, however, the calculated values represent satisfactory estimations of the experimental peak widths.

TABLE V

EXPERIMENTAL AND CALCULATED VALUES OF THE ELUTION CHARACTERISTICS OF THE N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOYLAMIDES IN ADSORPTION CHROMATOGRAPHY ON PORASIL A

Gradient elution, $\varkappa = 1.0$; $A = c_0 = 0$; $V_s = 38.5$ ml. Column dimensions: 400 × 3 mm; $V_m = 2.00$ ml. Adsorbent: Porasil A (60), 37–75 μm. Flow-rate of mobile phase (ethyl acetate–cyclohexane): 38.5 ml/h. Temperature: 20–22°. Detection: photometric, 440 nm. Chromatographed compounds: 1 = dimethylamide; 2 = diethylamide; 3 = di-*n*-propylamide; 4 = di-*n*-butylamide derivative; *ca.* 8 μg each. The numbers of the experiments agree with the numbers of the curves in Fig. 4. The experimental values represent the arithmetic means from three experiments.

| *Experiment No.* | $c_k$ | $B$ | *Compound* | $V'_{R(g)}$ *(ml)* | | $w_{(g)}$ *(ml)* | | $N_{(g)}$ | | $R_{s(g)}$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* |
| 5 | 0.25 | 0.0064935 | 1 | 62.65 | 56.68 | 8.17 | 5.22 | 1023 | 2022 | | |
| | | | 2 | 39.59 | 38.85 | 5.42 | 4.97 | 973 | 1081 | 3.39 | 3.50 |
| | | | 3 | 30.12 | 30.05 | 4.28 | 3.97 | 939 | 1043 | 1.95 | 1.96 |
| | | | 4 | 26.01 | 26.04 | 3.79 | 3.33 | 916 | 1134 | 1.02 | 1.10 |
| 6 | 0.5 | 0.012987 | 1 | 39.92 | 35.57 | 5.45 | 3.43 | 977 | 1919 | | |
| | | | 2 | 25.23 | 23.96 | 3.70 | 3.14 | 910 | 1094 | 3.21 | 3.53 |
| | | | 3 | 19.19 | 18.50 | 2.98 | 2.63 | 861 | 972 | 1.81 | 1.89 |
| | | | 4 | 16.57 | 16.00 | 2.66 | 2.30 | 837 | 980 | 0.93 | 1.01 |
| 7 | 1.0 | 0.025974 | 1 | 25.43 | 24.34 | 3.72 | 2.95 | 913 | 1276 | | |
| | | | 2 | 16.07 | 16.19 | 2.60 | 2.40 | 831 | 919 | 2.96 | 3.04 |
| | | | 3 | 12.22 | 12.47 | 2.14 | 1.92 | 775 | 909 | 1.62 | 1.72 |
| | | | 4 | 10.56 | 10.64 | 1.95 | 1.76 | 737 | 825 | 0.81 | 0.99 |
| 8 | 2.0 | 0.051948 | 1 | 16.20 | 16.42 | 2.62 | 2.50 | 830 | 869 | | |
| | | | 2 | 10.24 | 10.42 | 1.91 | 1.92 | 731 | 670 | 2.63 | 2.71 |
| | | | 3 | 7.79 | 7.98 | 1.61 | 1.86 | 675 | 461 | 1.39 | 1.29 |
| | | | 4 | 6.73 | 6.86 | 1.49 | 1.60 | 637 | 491 | 0.68 | 0.65 |

Tables IV–VII also give a comparison of 48 experimental and calculated values of the resolution, $R_{s(g)}$ (eqn. 11). The differences exceed 10% only for ten experimental values and 20% for one value. In most instances, the calculated resolution is slightly lower than the experimental values, which is connected with the systematic error in the calculated $w_{(g)}$ values. However, the agreement between the calculated and experimental values is better for $R_{s(g)}$ than for $w_{(g)}$.

Comparison of the experimental and calculated values of the plate number, $N_{(g)}$, shows that the calculation according to eqn. 10 can give a sufficiently good estimation of $N_{(g)}$ values. Some experimental values in Tables IV–VII differ considerably from the calculated values, which is probably caused by errors in the determination of $w_{(g)}$.

The results of the verification of eqns. 8–11 for $A = c_0$ differing from zero are presented in Table VIII. Here, the gradient functions are linear ($\varkappa = 1$) and the concentration of ethyl acetate in the mobile phase decreases during the elution ($c_k < c_0$). These functions are plotted in Fig. 4, V (Curves 18–20), for three different combinations of $c_0$ and $c_k$ ($A$ and $B$) values. The experimental and calculated values of $V'_{R(g)}$, $w_{(g)}$, $R_{s(g)}$ and $N_{(g)}$ are in good agreement.

TABLE VI

EXPERIMENTAL AND CALCULATED VALUES OF THE ELUTION CHARACTERISTICS OF THE N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOYLAMIDES IN ADSORPTION CHROMATOGRAPHY ON PORASIL A

Gradient elution, $\varkappa = 2.0$; $A = c_0 = 0$; $V_y = 38.5$ ml. Column dimensions: 400 × 3 mm; $V_m = 2.00$ ml. Adsorbent: Porasil A (60), 37–35 μm. Flow-rate of mobile phase (ethyl acetate–cyclohexane): 38.5 ml/h. Temperature: 20–22°. Detection: photometric, 440 nm. Chromatographed compounds: 1 = dimethylamide; 2 = diethylamide: 3 = di-*n*-propylamide; 4 = di-*n*-butylamide derivative; *ca.* 8 μg each. The numbers of the experiments agree with the numbers of the curves in Fig. 4. The experimental values represent the arithmetic means from three experiments.

| *Experiment No.* | $c_k$ | $B$ | *Compound* | $V'_{R(g)}$ *(ml)* | | $w_{(g)}$ *(ml)* | | $N_{(g)}$ | | $R_{s(g)}$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* |
| 9 | 0.125 | 0.0091831 | 1 | 75.56 | 74.17 | 6.16 | 4.17 | 2581 | 5338 | | |
| | | | 2 | 57.22 | 58.61 | 4.83 | 4.10 | 2460 | 3497 | 3.34 | 3.76 |
| | | | 3 | 48.48 | 50.62 | 4.19 | 3.46 | 2385 | 3701 | 1.94 | 2.11 |
| | | | 4 | 44.36 | 46.74 | 3.90 | 3.17 | 2327 | 3782 | 1.02 | 1.17 |
| 10 | 0.5 | 0.018366 | 1 | 43.76 | 42.60 | 3.85 | 2.95 | 2321 | 3657 | | |
| | | | 2 | 33.14 | 33.39 | 3.08 | 2.59 | 2163 | 2987 | 3.06 | 3.32 |
| | | | 3 | 28.07 | 28.71 | 2.72 | 2.27 | 2043 | 2929 | 1.75 | 1.92 |
| | | | 4 | 25.69 | 26.37 | 2.54 | 2.08 | 1995 | 2976 | 0.90 | 1.07 |
| 11 | 1.0 | 0.025974 | 1 | 33.30 | 32.56 | 3.09 | 2.59 | 2168 | 2849 | | |
| | | | 2 | 25.22 | 25.27 | 2.51 | 2.24 | 1976 | 2371 | 2.89 | 3.01 |
| | | | 3 | 21.36 | 21.55 | 2.23 | 1.95 | 1858 | 2334 | 1.63 | 1.77 |
| | | | 4 | 19.55 | 19.79 | 2.10 | 1.79 | 1791 | 2371 | 0.84 | 0.94 |
| 12 | 2.0 | 0.036732 | 1 | 25.34 | 24.66 | 2.52 | 2.30 | 1977 | 2150 | | |
| | | | 2 | 19.19 | 18.89 | 2.07 | 1.92 | 1784 | 1894 | 2.68 | 2.72 |
| | | | 3 | 16.26 | 16.16 | 1.86 | 1.63 | 1657 | 1986 | 1.49 | 1.53 |
| | | | 4 | 14.88 | 14.82 | 1.76 | 1.53 | 1591 | 1934 | 0.80 | 0.85 |

## CONCLUSIONS

The results show that eqn. 1 is well suited to describe the influence of the concentration of ethyl acetate in cyclohexane on the capacity ratios of N,N-dimethyl-*p*-aminobenzeneazobenzoylamides on columns packed with Porasil A. This equation can be used even when the concentration of ethyl acetate is expressed in volume ratios. Eqns. 4–6 for the retention volume, peak width and resolution in isocratic elution chromatography showed good validity in the system used and the eqns. 8–11 can be used successfully to predict the elution characteristics in gradient elution chromatography controlled by eqn. 3.

The validity of eqns. 4–11 was tested only in the chromatography of N,N-dimethyl-*p*-aminobenzeneazobenzoylamides on silica in cyclohexane–ethyl acetate as the mobile phase. As the chromatographic properties of real systems are expressed by means of eqn. 1 and further derivation of eqns. 4–11 is only a formal mathematical approach, it can be expected that these relationships will be equally useful in all of the chromatographic systems for which eqn. 1 can be used to describe the relationship between the capacity ratios of sample compounds and the concentration of the more efficient eluting component in a binary mobile phase.

TABLE VII

EXPERIMENTAL AND CALCULATED VALUES OF THE ELUTION CHARACTERISTICS OF THE N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOYLAMIDES IN ADSORPTION CHROMATOGRAPHY ON PORASIL A

Gradient elution, $\varkappa = 4.0$; $A = c_0 = 0$; $V_s = 38.5$ ml. Column dimensions: 400 × 3 mm; $V_m = 2.00$ ml. Adsorbent: Porasil A (60), 37–75 $\mu$m. Flow-rate of mobile phase (ethyl acetate–cyclohexane): 38.5 ml/h. Temperature: 20–22°. Detection: photometric, 440 nm. Chromatographed compounds: 1 = dimethylamide; 2 = diethylamide; 3 = di-*n*-propylamide; 4 = di-*n*-butylamide derivative; *ca.* 8 $\mu$g each. The numbers of the experiments agree with the numbers of the curves in Fig. 4. The experimental values represent the arithmetic means from three experiments.

| *Experiment No.* | $c_k$ | *B* | *Compound* | $V'_{R(a)}$ *(ml)* | | $w_{(a)}$ *(ml)* | | $N_{(a)}$ | | $R_{s(a)}$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* |
| 13 | 0.25 | 0.018366 | 1 | 51.62 | 50.91 | 2.77 | 2.66 | 6146 | 6330 | | |
| | | | 2 | 44.18 | 43.57 | 2.47 | 2.40 | 5757 | 5769 | 2.84 | 2.82 |
| | | | 3 | 40.27 | 39.94 | 2.31 | 2.24 | 5529 | 5609 | 1.64 | 1.64 |
| | | | 4 | 38.32 | 37.98 | 2.23 | 2.02 | 5406 | 6268 | 0.86 | 0.92 |
| 14 | 0.5 | 0.021842 | 1 | 44.30 | 43.82 | 2.48 | 2.34 | 5740 | 6135 | | |
| | | | 2 | 37.93 | 37.56 | 2.22 | 2.08 | 5351 | 5788 | 2.71 | 2.82 |
| | | | 3 | 34.57 | 34.35 | 2.08 | 1.95 | 5129 | 5560 | 1.56 | 1.59 |
| | | | 4 | 32.89 | 32.62 | 2.01 | 1.89 | 5008 | 5368 | 0.82 | 0.90 |
| 15 | 1.0 | 0.025974 | 1 | 38.03 | 37.53 | 2.22 | 2.21 | 5378 | 5119 | | |
| | | | 2 | 32.56 | 32.30 | 2.00 | 1.95 | 4965 | 4951 | 2.59 | 2.50 |
| | | | 3 | 29.67 | 29.48 | 1.89 | 1.86 | 4685 | 4583 | 1.49 | 1.48 |
| | | | 4 | 28.23 | 27.87 | 1.83 | 1.73 | 4562 | 4770 | 0.77 | 0.89 |
| 16 | 2.0 | 0.030888 | 1 | 32.64 | 32.40 | 2.01 | 2.08 | 4740 | 4376 | | |
| | | | 2 | 27.94 | 27.65 | 1.81 | 1.76 | 4576 | 4541 | 2.46 | 2.47 |
| | | | 3 | 25.47 | 25.31 | 1.71 | 1.63 | 4333 | 4492 | 1.40 | 1.38 |
| | | | 4 | 24.23 | 24.06 | 1.66 | 1.53 | 4202 | 4642 | 0.74 | 0.79 |
| 17* | 0.25 | 0.018366 | 1 | 36.22 | 35.35 | 2.77 | 2.72 | 3046 | 3017 | | |
| | | | 2 | 28.78 | 28.29 | 2.47 | 2.27 | 2485 | 2849 | 2.84 | 2.82 |
| | | | 3 | 24.87 | 24.54 | 2.31 | 2.24 | 2165 | 2246 | 1.64 | 1.66 |
| | | | 4 | 22.92 | 22.42 | 2.23 | 2.08 | 1998 | 2205 | 0.86 | 0.98 |

* Gradient function is given by curve 13, but the elution was started from the point corresponding to 15.40 ml of the eluate, where the concentration of ethyl acetate is still virtually zero.

Eqns. 8–11 for the elution characteristics in gradient elution chromatography can also be used with systems in which the two components of the mobile phase are mixed in volume ratios that differ significantly from the respective mole ratios. In such an instance, the gradient function describing the dependence of the volume ratio of the more efficient eluting component in the mobile phase on the volume of the eluate should be constructed in such a way that the mole ratio of this component would follow a function defined by eqn. 3.

The good agreement between the constants $k'_0$ and $n$ evaluated from the experiments using gradient elution and the values obtained in isocratic elution chromatography suggests the possibility of using gradient elution chromatography for the determination of these constants. This method could avoid lengthy experiments using mobile phases that have a poor eluting strength in isocratic elution.

The relationships derived in Parts I and II[1,2] and verified in this paper are likely to form a reliable basis for calculations of elution characteristics in isocratic and gradient elution chromatography and, even more important, for the prediction of the

TABLE VIII

EXPERIMENTAL AND CALCULATED VALUES OF THE ELUTION CHARACTERISTICS OF THE N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOYLAMIDES IN ADSORPTION CRHOMATOGRAPHY ON PORASIL A

Gradient elution, $\varkappa = 1.0$; $c_k = 0$. Column dimensions: 400 × 3 mm; $V_m = 2.00$ ml. Adsorbent: Porasil A (60), 37–75 $\mu$m. Flow-rate of mobile phase (ethyl acetate–cyclohexane): 38.5 ml/h. Temperature: 20–22°. Detection: photometric, 440 nm. Chromatographed compounds: 1 = dimethylamide; 2 = diethylamide; 3 = di-*n*-propylamide; 4 = di-*n*-butylamide derivative; *ca.* 8 $\mu$g. each The numbers of the experiments agree with the numbers of the curves in Fig. 4. The experimental values represent the arithmetic means from three experiments.

| *Experiment No.* | $A = c_0$ | $B$ | $V_y$ (ml) | *Compound* | $V'_{R(g)}$ (ml) | | $w_{(g)}$ (ml) | | $N_{(g)}$ | | $R_{s(g)}$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* |
| 18 | 1.0 | −0.025974 | 38.5 | 1 | 4.61 | 4.48 | 2.46 | 2.56 | 116 | 102 | 1.96 | — |
| | | | | 2 | 1.14 | 1.53 | 1.08 | — | 135 | — | 0.65 | — |
| | | | | 3 | 0.51 | 0.95 | 0.86 | — | 136 | — | 0.22 | — |
| | | | | 4 | 0.33 | — | 0.80 | — | 136 | — | | |
| 19 | 0.5 | −0.025974 | 19.25 | 2 | 5.24 | 4.67 | 3.16 | 3.36 | 84 | 63 | 1.39 | — |
| | | | | 3 | 2.03 | 2.01 | 1.46 | — | 122 | — | 0.57 | — |
| | | | | 4 | 1.29 | 1.27 | 1.15 | — | 131 | — | | |
| 20 | 0.25 | −0.010390 | 24.06 | 3 | 10.18 | 9.61 | 7.02 | 7.44 | 48 | 39 | 0.93 | 0.83 |
| | | | | 4 | 5.46 | 5.06 | 3.10 | 3.46 | 93 | 67 | | |

optimum parameters of a concentration gradient necessary for the resolution of particular sample components. This optimization of gradient elution is the subject of our current investigations.

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